INCREASED PERCENTAGES OF TUMOR NECROSIS FACTOR-α⁺/INTERFERON-T⁺LYMPHOCYTES AND CALPROTECTIN⁺/TUMOR NECROSIS FACTOR-A⁺ MONOCYTES IN PATIENTS WITH ACUTE KAWASAKI DISEASE

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***In vivo* exposure to microorganisms resident in the oral cavity is considered as a possible cause of Kawasaki disease (KD), and some epitopes derived from streptococci display homology with Factor H of Complement. Additionally, calprotectin, a major calcium binding protein released by neutrophils and activated monocytes, could be directly involved in endothelial damage occurring in KD. The aim of our study is to evaluate the percentages of IFN-γ⁺ and/or TNF-α⁺ lymphocytes and double positive calprotectin/TNF-α monocytes (CD14⁺) after *in vitro* stimulation with streptococcal- and/or Factor H-derived peptides, in patients with acute KD. Peripheral Blood Mononuclear Cells (PBMCs) obtained from KD patients and febrile controls were stimulated *in vitro* with peptides. After culture, cells were collected, stained with fluorochrome-labelled monoclonal antibodies against CD3, CD14, calprotectin, IFN-γ and TNF-α, and cytofluorimetric analyses were performed. Our results showed increased percentages of TNF-α⁺/IFN-γ⁺ lymphocytes in KD patients in respect to controls when PBMCs were stimulated with streptococcal or Factor H-derived epitopes. In addition, also calprotectin⁺/TNF-α⁺ monocytes from KD patients were activated after PBMC *in vitro* stimulation. These findings lead us to speculate that some peptides, derived from oral streptococci and cross-reactive with the human Factor H of Complement, could induce lymphocyte and monocyte activation potentially involved in the pathogenesis of KD. Our results should be confirmed by further studies enrolling more patients and controls than those analyzed in our study.**

Kawasaki disease (KD) is an acute febrile illness that primarily arises in infancy and early childhood characterized by diffuse vasculitis of medium-sized arteries. Clinical features consist of fever accompanied by lymphadenopathy, skin rash, conjunctivitis, oropharyngeal mucosal changes and extremity changes (1). Diagnostic criteria, used in clinical approach to patients may, however, be inadequate, and early diagnosis frequently remains challenging, with high risk of coronary damage. In fact, in most cases it is a limited illness resolving in few days after fever onset, but without appropriate intervention coronary artery aneurysms can develop in about 15-25% of the patients (1). The acute phase of KD is characterized by a deficiency of suppressor T cells, marked activation of the immune

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system and increased secretion of pro-inflammatory cytokines by immune effector cells, such as TNF-α, IFN-γ, IL-1β, IL-6 (1-3). Another hematological alteration commonly found in KD is the increase of peripheral blood monocytes (3). Moreover, some studies reported the increased production of TNF-α, and in particular its role in the inflammation of arteries, as immunological marker of acute phase of KD. In fact, infliximab and etanercept, monoclonal antibodies blocking the biological activity of TNF-α, were used for refractory KD patients and were reported to be effective and safe (4, 5). An efficient therapy, based on intravenous infusion of high doses of immunoglobulins (IVIG), has been found to effectively reduce the inflammation and the incidence of coronary artery lesions (6).

Recently, it has been shown that myeloid-related proteins (MRP-8 and MRP-14) of S100- family, the major calcium-binding proteins secreted by activated neutrophils and monocytes, control myeloid cell function and inflammation, in part, through activation of Toll-like receptor-4. The MRP-8/MRP-14 complex binds specifically to human microvascular endothelial cells through heparan sulfate proteoglycans and novel carboxylated N-glycans, contributing to cause inflammation in acute vascular lesions occurring in several diseases such as KD (7). Moreover, there is increasing evidence that streptococci infections and the upregulation of calprotectin, one of the major calcium-binding proteins, may directly and indirectly induce not only inflammation but also modifications affecting the microvascular wall in acute vasculitis syndromes (2, 7).

Several years ago, scientists identified as a possible cause of KD the exposure to microbial agents derived from organisms resident in the oral cavity such as *Streptococcus (S.) oralis, S. mitis, S. sanguinis* and *S. parasanguinis* (8). Additionally, it has been shown that phosphoglyceratemutase (gpm1) of *Candida albicans* and *Saccharomyces cerevisiae* are Factor H- and FHL-1-binding protein (9).

Following this background, we analysed the percentages of intracellular calprotectin and/or TNF-α positive monocytes and the activation of T lymphocytes after *in vitro* exposure to several commensal streptococci- and/or Factor H-derived peptides, as a novel immune correlate of inflammation and possible cause of endothelium damage mainly occurring in the acute phase of KD. To evaluate the association between the degree of inflammation and the alteration of micro-vascular wall, we analysed TNF-α and IFN-γ positive lymphocytes and the production of calprotectin and TNF-α in $CD14^+$ cells, probably involved in the endothelial damage suggestive of KD after lymphocyte peptide specific activation.

MATERIALS AND METHODS

Patients

In this study we enrolled 8 children (5M, 3F), affected by Kawasaki disease, diagnosed following international criteria (10) and recruited during the years 2010-2011. At diagnosis, their age ranged from 4 to 45 months. A parents' informed consent was obtained for patients and for febrile controls, as suggested by the bioethics committee of Meyer Hospital, Florence and Paolo Giaccone University Hospital, Palermo. All the KD patients, in addition to fever and the classic criteria, showed signs of cardiac involvement, specifically pericarditis in two cases and coronary lesions in the other six. Only two of them improved with a single cycle of intravenous immunoglobulins, while the other six needed a second cycle; one of them also needed intravenous corticosteroids and infliximab. As this patient did not respond to IVIG therapy, we excluded the results obtained by the PBMCs of this patient from the patient data. Heparinised blood samples from KD patients were collected before IVIG therapy. Laboratory tests showed in all cases anaemia, leukocytosis (present also in controls) thrombocytosis and elevation of acute phase indexes. All of the children, ultimately, had total remission of clinical signs and symptoms, and also had resolution of coronary alterations. Febrile patients with documented viral or bacterial infections (4M and 4F, age-matched with KD patients) were also enrolled as controls. All patients and controls were typed for HLA DR B1 alleles resulting heterozygous by Low Resolution SSO test (One Lambda, California, USA) as shown in Table I.

Peptides

A total of 4 peptides derived from the sequence of the proteins of *Streptococcus sanguinis* (p28,#1(or #2010),#4) and chain A, solution structure of human complement factor H (#42), were acquired from LIONEX Diagnostics & Therapeutics (Braunshweig, Germany). Peptide #1 (EKSWRLNERHYGGLTGKN

Table I. *Typing results of patients and febrile controls obtained with low resolution kit (One Lambda, California, USA).*

Patient 1	DR B1 0301/DR 0401	Febrile Control 1	DR 0401/DR 0301
Patient 2	DR B1 0301/ DR 0401	Febrile Control 2	DR 0701/DR 0301
Patient 3	DR 0401/DR 0101	Febrile Control 3	DR 1501/DR 0901
Patient 4	DR 0701/DR 0301	Febrile Control 4	DR 0801/DR 1101
Patient 5	DR 0101/DR 0701	Febrile Control 5	DR 0401/DR 0301
Patient 6	DR 1101/DR 0901	Febrile Control 6	DR 0101/DR 0301
Patient 7	DR 1501/DR 1101	Febrile Control 7	DR 1101/DR 1501
Patient 8	DR 0801/DR 1501	Febrile Control 8	DR 0401/DR 0701

KAEAAE), peptide #4 (gpm1 like-molecules derived from *S. sanguinis*) (YDVLPPAMPRDDEYSAHTDRRY AS), peptide #42 (chain A, solution structure of human complement factor H), p28 (LNDTLDYERHDGYESNT GSTTGS) and #42 (LNDTLDYECDGYESNTGSTGS). Analysis of peptide sequences showed high homology between p28 and the human complement factor H-derived peptide #42 [identities = 20/23aminoacids (87%); http: //blast.ncbi.nlm.nih.gov/Blast]. We used a bioinformatics software analysis (through SYFPEITHY website) to detect the highest binding scores of the proteins with the most frequent HLA DRB1 alleles in Caucasians. The peptides with maximal binding capacity with the most frequent HLA DRB1 alleles were p28 and #42 (derived from chain A of the human Complement factor H), that display a high degree of homology. These peptides were chosen also because they bind with high score many HLA-DRB1 alleles frequently detected in Caucasians. In particular, p28 epitopes display the highest score of binding with HLA DRB1 alleles than the other epitopes used. Because the sequences of these proteins show a high degree of homology with some "immunodominant" epitopes of *S. sanguinis,* we analysed *in vitro* responses of Peripheral Blood Mononuclear Cells (PBMCs) to these epitopes. We used these proteins and peptides to understand whether some residues are involved in lymphocyte activation. The purity rate of peptides was 95% and their homogeneity was confirmed by analytical reverse-phase high-performance liquid chromatography, mass spectroscopy, and aminoacid composition analysis.

Cell cultures and intracellular staining

PBMCs were isolated from heparinized blood by centrifugation on Ficoll-Hypaque (Pharmacia). The medium used throughout was RPMI 1640 (Invitrogen Life Technologies) supplemented with 10% heat-inactivated fetal cow serum (FCS), 2 mM L-glutamine, 20 mM HEPES, 100U/ml penicillin, 100μg/ml streptomycin, 5 x 10^{-5} M 2-ME. PBMCs (10^6/ml) were stimulated with peptides (25μg/ml, final concentration) for 16 h at 37°C in 5% CO_2; 3 μM monensin (Sigma, Massachussetts, USA) was added after 2 h of incubation as Golgi blocker.

PBMCs cultured without any stimulus (only complete medium) were used as negative control and unstimulated PBMCs were evaluated to assess nonspecific/background cytokine production. These cells were also cultured for 16 h at 37°C in 5% CO_2 and 3 μM monensin was added after 2 h of incubation, together with other cells, as above-mentioned. All the cells were then harvested, washed and stained with anti-CD3-FITC (cloneUCHT1,BD Pharmingen, San Jose`, CA, USA), anti-CD4-PerCP5.5 (clone RPA-T4, BD Pharmingen, San Jose`, CA, USA), anti-CD8-PerCP5.5 (clone RPA-T8, BD Pharmingen, San Josè, CA, USA) and anti-CD14-PerCP5.5 (clone 61D3, eBiosciences, San Diego, CA, USA), both diluted in incubation buffer, containing PBS, 1% FCS and 0.1% Na azide, for 30 min at 4°C. Cells were washed twice in PBS-1% FCS and then fixed and permeabilized (Fix and Perm kit, BD Pharmingen, San Josè, CA, USA) to perform the intracellular staining with anti-TNF-α-PE (clone J1D9, Ancell Corporation, Bayport, USA), anti-IFN-γ-APC (clone B2, BD Pharmingen, San Josè, CA, USA), anti-calprotectin-FITC (clone 27E10, Hycult biotech, Uden, The Netherlands) and isotype-matched control mAb (eBiosciences, San Diego, CA). 100.000 events were acquired and analysed for each sample by FACS Calibur.

Statistics

Values obtained from intracellular staining of PBMCs cultured only with RPMI plus 10% FCS were used as negative controls and subtracted from those obtained from PBMCs stimulated with peptides. Student's *t* and Mann-Whitney tests were used to determine statistical significances comparing data obtained from age-matched patients and controls. Values of $p<0.05$ were considered significant.

RESULTS

Our results showed increased mean percentages of CD3 positive cells producing IFN-γ and/or TNF-α in KD patients in respect to febrile controls when stimulated with peptides. The highest percentages of activated lymphocytes (IFN-γ^+ and/or TNF-α^+) were found after stimulation with p28 and #42 (Fig.1, A and B), probably it may depend on the affinity and avidity of a single epitope. The mean percentages of IFN-γ^+ and/or TNF-α^+CD3$^+$ were statistically significant ($p< 0.05$) among stimulated cells of KD patients and controls (Fig. 1A). To characterize the phenotype of IFN-γ^+ and/or TNF-α^+ CD3$^+$ T cells, CD4 or CD8 expression on these cells was analyzed by triple staining of these cells. Mainly CD3$^+$ CD4$^+$IFN-γ^+ and/or TNF-α^+ cells were detected (91%) (data not shown). Fig. 1B shows representative dot plot analysis of CD3$^+$ cells from a patient and a control, after culture with p28.

Additionally, patients with acute KD showed increased mean percentages of calprotectin$^+$/CD14$^+$ cells in the same culture conditions. On the contrary, age-matched febrile controls did not show any increase of intracellular calprotectin in CD14$^+$ cells (Fig. 2A). The differences between the mean percentages of CD14$^+$/calprotectin$^+$ cells from patients and controls were statistically significant. A representative dot plot analysis of CD14$^+$/calprotectin$^+$ obtained from one KD patient and one febrile control are shown in Fig. 2B.

We also analysed the Mean Fluorescence Intensity (MFI) of intracellular calprotectin expressed in CD14$^+$ and we found higher MFI in patients respect to control for all the peptides tested (Fig.3).

These results suggest an important role for activated CD3 positive cells, specifically stimulated with peptides derived from *S. sanguinis* and/or Factor-H, in the expansion of calprotectin$^+$/CD14$^+$ monocytes. Considering this subset of monocytes as a possible player in the pathogenesis of endothelial damage, we also analysed TNF-α production in calprotectin positive monocytes. In fact, detection of a single molecule, such as TNF-α or calprotectin, does not fully reflect the functional potential of the cells involved in the pathogenesis of the disease. Statistically significant higher mean percentages of TNF-α^+/calprotectin$^+$/CD14$^+$ cells were observed ($p< 0.05$) after *in vitro* stimulation of PBMCs with p28 and #42 peptides in all patient samples tested when compared to those obtained from febrile controls (Fig 2C). A representative dot plot analysis of calprotectin/TNF-α positive cells, gated on CD14$^+$ population, of one KD patient and febrile control is shown in Fig.2D.

Regarding the strength of PBMCs' response to the epitopes, we detected a maximal activation of lymphocytes and of double positive calprotectin/TNF-α CD14$^+$ cells, when p28 and #42 (degree of homology of 87%) were used. These data demonstrate, for the first time to our knowledge, that calprotectin$^+$/TNF-α^+ CD14$^+$ cells could be associated with the acute phase of KD, suggesting a pathogenetic role for this subset in this vasculitis.

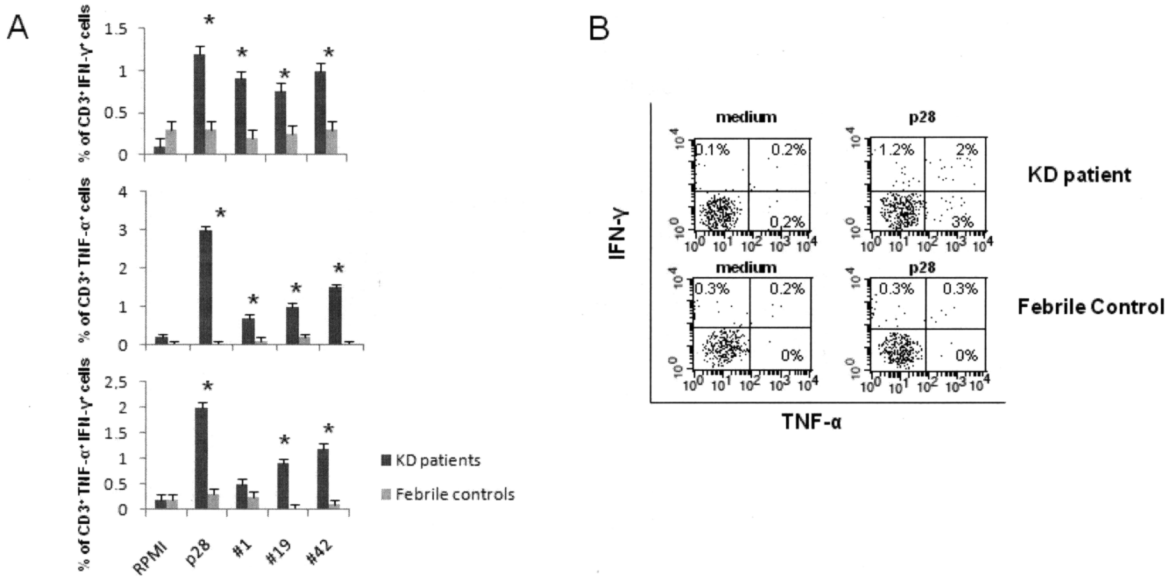

Fig. 1. ***A)*** *IFN-γ and/or TNF-α production by CD3⁺ lymphocytes after PBMC culture with specific peptides. The histograms show highest mean percentages of activated lymphocytes (producing IFN-γ and/or TNF-α)* in KD patients *in respect to controls, (*p<0.05).* ***B)*** *Dot plot analysis of IFN-γ/TNF-α* positive cells among CD3 positive lymphocytes of one representative KD patient and one *age-matched febrile control. Error bars represent standard deviation.*

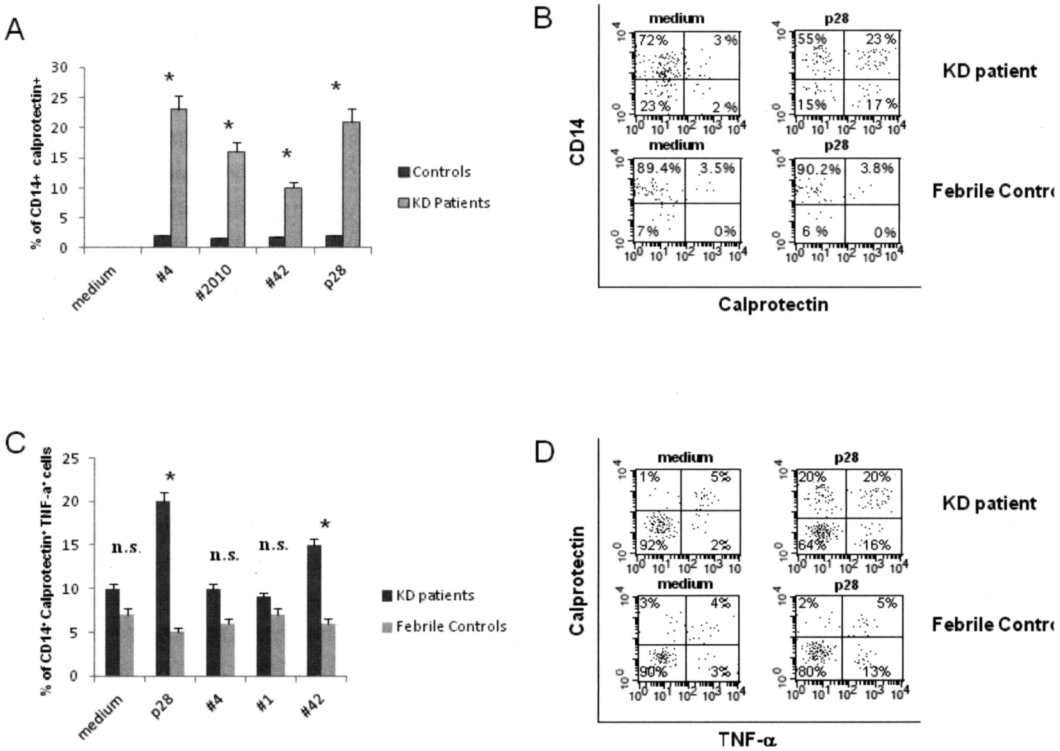

Fig. 2. ***A)*** *Comparison of the frequencies of CD14⁺/calprotectin⁺ cells in peripheral blood from KD patients with acute disease before starting therapy and febrile controls. The histograms show highest mean percentages of intracellular calprotectin in patients with acute KD; the lowest mean percentages were found in controls, * p<0.05.* ***B)*** *Dot plot analysis of CD14⁺/calprotectin⁺ cell populations of one representative KD patient and one febrile control.* ***C)*** *Increased mean percentages of double positive calprotectin/TNF-α CD14⁺ cells after peptide-specific stimulation in patients in respect to febrile controls, * p<0.05.* ***D)*** *Dot plot analysis of CD14⁺calprotectin/TNF-α positive cell populations of one representative KD patient and one age-matched febrile control. Error bars represent standard deviations.*

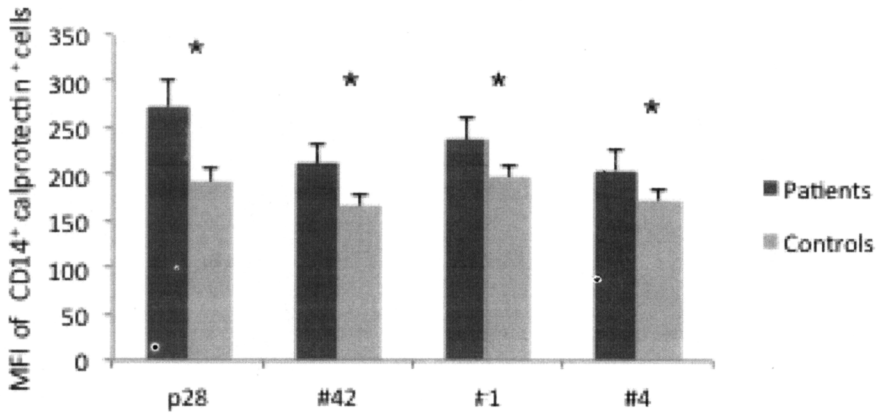

Fig. 3. *An MFI analysis of CD14$^+$calprotectin$^+$ cells was assessed to compare the values between patients (dark grey columns) and controls (grey columns). All the differences were statistically significant ($p<0.05$).*

DISCUSSION

CD14$^+$ cells play a critical role in several inflammatory disorders (11, 12), but their role has gained increasing attention also in KD pathogenesis (13). In the present study, we investigated lymphocyte activation after stimulation of PBMCs with different peptides derived from *S.sanguinis* and Factor H of Complement, and calprotectin and TNF-α double positive monocytes obtained from KD patients and febrile controls. Our results showed CD3 positive cell activation after culture with peptides derived from oral streptococci in KD patients in respect to the control group. We also demonstrated, for the first time, that KD patients have high percentages of calprotectin$^+$ cells and double positive calprotectin/TNF-α CD14$^+$ cells. These findings lead us to speculate that some peptides derived from oral streptococci and the human Factor H of Complement can be involved in the pathogenesis of KD, probably through production of immunobiologically active extracellular products (i.e. IFN-γ and TNF-α) by lymphocytes that finally promote monocyte activation. Our data also suggest that double positive calprotectin/TNF-α positive monocytes could play an important role in triggering the vasculitis in acute KD. Regarding the strength of PBMCs' response to the epitopes, we detect a maximal activation of lymphocytes, and the sequential increase (probably due to IFN-γ and/or TNF-α release) of double positive calprotectin/TNF-α CD14$^+$ cells, when p28 and #42 (degree of homology of 87%) were used. These two epitopes could have aminoacidic residues providing anchoring sites for MHC and/or TCRs binding, preferentially involved in the activation of T cell. These data demonstrate, for the first time to our knowledge, that calprotectin$^+$/TNF-α$^+$ CD14$^+$ cells could be associated to the acute phase of KD, suggesting a pathogenetic role of this subset in this vasculitis. Clearly we cannot exclude that other cytokines, such as IL-1, IFN-γ and/or IL-6 could be secreted by activated monocytes causing, in synergy with calprotectin and TNF-α, endothelial damage. Thus, a microbial agent, cross-reacting with endogenous Factor H of Complement, can stimulate the production of calcium-binding proteins that could participate, synergistically with TNF-α, to the vasculitis. Our data strengthen Foell's results (2), suggesting that calcium-binding proteins could serve as a novel target for future therapeutic intervention in inflammatory disorders, and also that calprotectin/TNF-α positive monocytes can be used as markers to monitor disease course and therapy.

In conclusion, calprotectin$^+$/TNF-α$^+$ monocytes, induced by IFN-γ$^+$/TNF-α$^+$ lymphocytes activated by p28 and #42 epitopes, could represent an important effector cell subset involved in the generation of acute KD. We hypothesize that this cell subset could be used

as a marker of acute KD. Novel immunotherapeutic approaches, based on the combination of anti-calprotectin and anti-TNF-α agents, could be suggested for an efficient KD therapy. Moreover, other studies will be necessary to better characterize these cells and their role in the disease.

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